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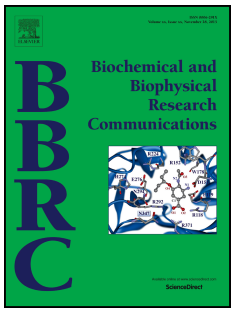

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Carboxypeptidase X-1 (CPX-1) is a secreted collagen-binding glycoprotein

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Abstract

Carboxypeptidase X-1 (CPX-1) is an atypical member of the carboxypeptidase (CP) family of proteins involved in a variety of physiological and pathological processes. However, unlike most other family members CPX-1 lacks catalytic activity making its biological function unclear. CPX-1 contains a 160 amino acid discoidin domain (DSD) that serves as a binding domain in other proteins prompting us to investigate a putative functional role for this domain in CPX-1. Sequence alignment confirmed the overarching homology between the DSD of CPX-1 and other DSDs whilst more detailed analysis revealed conservation of the residues known to form the collagen-binding trench within the DSD of the discoidin domain receptors (DDRs) 1 and 2. Biochemical characterisation of transiently expressed human CPX-1 revealed that CPX-1 was secreted in an N-glycosylation-dependent manner as treatment with the N-glycosylation inhibitor tunicamycin inhibited secretion concomitant with a reduction in CPX-1 mobility on Western blot. Using a collagen pull-down assay we found that secreted CPX-1 bound collagen and this appeared independent of N-glycosylation as treatment with PNGaseF did not affect binding. Further analysis under non-reducing and reducing (+DTT) conditions revealed that CPX-1 was secreted in both monomeric and dimeric forms and only the former bound collagen. Finally, mutation of a key residue situated within the putative collagen-binding trench within the DSD of CPX-1 (R192A) significantly reduced secretion and collagen-binding by 40% and 60%, respectively. Collectively these results demonstrate that CPX-1 is a secreted collagen-binding glycoprotein and provide a foundation for future studies investigating the function of CPX-1.

Keywords

Carboxypeptidase, Discoidin Domain, Collagen

Introduction

The Carboxypeptidase (CP) superfamily of enzymes typically performs a broad range of functions by removing C-terminal amino acids from proteins and peptides. As such, they play key roles in processes including the biosynthesis of neuropeptides and peptide hormones as well as in the degradation of proteins within the digestive tract. The M14 family of metallocarboxypeptidases is the largest family of CPs and most, but not all, are active as peptidases [1]. The carboxypeptidase E (CPE) subfamily consists of eight members, of which five are enzymatically active peptidases [2]. The three members that lack catalytic activity, CPX-1, CPX-2 and aortic carboxypeptidase-like protein/adipocyte enhancer binding protein 1 (ACLP/AEBP1) form a discrete subset [2]. Their CP domains show the highest degree of homology within the family with each lacking one or more residues critical for enzymatic activity and/or substrate binding [2,3,4]. Furthermore, each contains an N-terminal signal peptide followed by a discoidin domain (DSD), a domain not present in the other family members [2]. DSDs, which are also called factor 5/8 type C domains [5], are 150-160 amino acid domains present in a large number of proteins with a wide range of functions [6]. They typically serve as binding domains, binding to a diverse array of molecules including growth factors, phospholipids and lipids, galactose and collagen, and are implicated in a range of physiological and pathophysiological processes [6,7,8].

CPX-1 was first identified and characterised almost 20 years ago when it was shown to be a secreted protein subject to N-glycosylation [3]. Empirical evidence suggests it lacks catalytic activity [3] and this finding is consistent with sequence alignments which reveal substitution of two residues critical for substrate binding (R117 and Y248 in CPB are replaced by Val and His residues, V408 and H558, in CPX-1) [2,3]. Thus, the molecular role and biological function of CPX-1 are unclear. One study has described a role for CPX-1 in osteoclastogenesis [9]. A proteomics based approach revealed that CPX-1 levels were transiently increased upon RANKL-stimulated induction of differentiation of pre-osteoclasts with a subsequent decrease in CPX-1 levels as the cells progressed to mature osteoclasts [9]. Additional investigations demonstrated that constitutive overexpression of CPX-1 inhibited the formation of multinucleated osteoclasts, but not the generation of mononuclear pre-osteoclasts, prompting the suggestion that tightly-

coordinated expression of CPX-1 may be required for the progression of pre-osteoclasts to osteoclasts [9]. Whilst establishing a putative role for CPX-1 in the efficient differentiation of osteoclasts these investigations provided no insight into the underlying molecular mechanisms.

At the molecular level CPX-1 is a modular protein containing a classic signal peptide, a DSD and a catalytically inactive CP domain [3] (see Fig 1A). Given the absence of enzymatic activity and the recognised functional and biological importance of the DSDs in a range of other proteins [6,7,8] we reasoned that increased understanding of the putative role of the DSD in CPX-1 would help direct future studies investigating the biological function of CPX-1. To this end, we have employed a complementary range of computational, cellular and biochemical approaches that show that CPX-1 is a secreted, collagen-binding glycoprotein.

Materials and methods

Reagents and antibodies

Unless otherwise stated, general reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and tissue culture reagents were from Invitrogen (Mount Waverley, Victoria, Australia). CPX-1 antibody was from R&D Systems (Minneapolis, MN, USA). β-tubulin antibody was from Sigma-Aldrich. Secondary antibodies were from Rockland Immunochemicals (Limerick, PA, USA).

In silico approaches

NetNGlyc 1.0 was used to predict putative N-Glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/)[10,11]. CLUSTAL multiple sequence alignment was used to perform sequence alignments (www.drive5.com/muscle/)[12].

CPX-1 overexpression

Overexpression of human CPX-1 was performed using a CPX-1 expression vector from GeneCopoeia (Rockville, MD, USA). Transfection into Chinese hamster ovary (CHO) cells or Human embryonic kidney cells (HEK) cells was performed using Lipofectamine and PLUS

Reagents according to the manufacturer's instructions (Invitrogen). Endogenous CPX-1 was undetectable by Western blot in both cell lines (not shown).

Site directed mutagenesis

Site directed mutagenesis was performed to generate CPX-1_{R192A} (R192 to Ala) using the QuikChange mutagenesis kit according to the manufacturer's instructions (Invitrogen). Sequence confirmation of CPX-1_{R192A} was performed using Sanger sequencing.

In vitro pull-down assay

Binding conditions were optimised in preliminary studies to reduce non-specific binding to IgG-agarose beads (which were used throughout as a negative control). Prior to use collagen-agarose beads, featuring collagen III, (Sigma) were washed once with PBS and twice with pull-down buffer (150mM NaCl, 0.1% Triton X-100) and resuspended to give a 1:1 slurry of bead:buffer by volume. CPX-1 in conditioned, serum free medium was prepared for use by concentrating (1 in 10-20) using Amicon Ultra Centrifugal Filters (30kDa) according to the manufacturer's instructions (Millipore, Victoria, Australia). This (5-10µl) was then incubated with collagen-agarose beads (30-50 µl) in pull-down buffer (total volume of 500µl) overnight at 4°C or for 3h at 37°C with constant mixing. Samples were centrifuged at 3,000 rpm for 30s and supernatant (unbound fraction) was transferred to a new tube and pellet (bound fraction) was washed 4 times with pull-down buffer. Fractions were analysed by Western blot.

Western blotting

Western blotting was carried out using SDS-PAGE as described [13].

Statistical analyses

Data are presented as mean ± SEM. Significance was determined using students t-test with statistical significance defined as $p < 0.05$.

3. Results

CPX-1 is secreted in an N-glycosylation dependent manner

CPX-1 has been reported to be secreted in an N-glycosylated form [3]. *In silico* analysis revealed 4 putative N-glycosylation sites in human CPX-1 including one situated within the DSD (Fig 1A). We reasoned that elucidation of the characteristics of N-glycosylation would increase our understanding of CPX-1 at the molecular level. As N-glycosylation has been shown to be essential for efficient secretion of some but not all N-glycosylated proteins [14] we first determined whether N-glycosylation was required for efficient secretion of CPX-1. Human CPX-1 was transiently expressed in CHO cells and intracellular and secreted CPX-1 was analysed by Western blot. The predicted molecular weight of CPX-1 (based on amino acid sequence) is 80kDa. Intracellular CPX-1 migrated at 80 and 100kDa whilst secreted CPX-1, present in conditioned medium, migrated predominantly at 120kDa, respectively (Fig 1B). These observations are consistent with CPX-1 undergoing a series of post-translational modifications (PTMs) during transit through the secretory pathway.

We used tunicamycin, an inhibitor of the enzyme N-acetylglucosamine phosphotransferase which catalyses the first step of N-glycosylation, to investigate a putative role for N-glycosylation in the secretion of CPX-1. Incubation of CPX-1 expressing cells with tunicamycin for 3h or 24h selectively reduced the intensity of the 100kDa band, with only the 80kDa form of intracellular CPX-1 detected after 24h (Fig 1C). Moreover, 24h incubation with tunicamycin completely abolished secretion of CPX-1 (Fig 1D). We used PNGaseF, which cleaves N-linked oligosaccharides, to complement and extend these observations. Treatment of cell lysates with PNGaseF resulted in conversion of the intracellular 100kDa band to 80kDa (Fig 1E). Interestingly, treatment of conditioned media with PNGaseF resulted in conversion of the 120kDa band to a band that migrated at around 100kDa but not 80kDa (Fig 1F). Collectively these results demonstrate that N-glycosylation of CPX-1 is essential for efficient secretion and also raise the possibility that secreted CPX-1 may be subject to additional, PNGaseF-independent PTM.

In silico analysis suggests the DSD of CPX-1 may bind collagen

In other proteins the DSD typically serves as a binding domain, where its biological role is determined predominantly by the nature of the binding partner [6]. Given the CP domain of

CPX-1 is catalytically inactive [3] characterisation of the DSD may be expected to provide useful insights into the putative function of CPX-1. To address this we performed an alignment of the DSDs from several proteins including the other two CPE subfamily members that lack catalytic activity, namely CPX-2 and ACLP, the collagen-binding DDRs, DDR1 & DDR2, the phospholipid-binding Coagulation Factors V and VIII, and the semaphorin receptor Neuropilin-1 (Fig 2). There was relatively modest homology at the primary sequence level with 20 identical and 32 conserved residues, consistent with previous analyses [6]. As expected the greatest homology with CPX-1 was observed for CPX-2 and ACLP, sharing 58.9 and 53.2% identity. The other DSDs shared between 35-29% identity with the CPX-1 DSD showing greatest homology with the DSDs of the collagen-binding DDRs (Fig 2). The molecular details of the DSD–collagen interaction have been elucidated through a combination of NMR studies [15], resolution of the crystal structure of the DDR2-DSD–collagen complex [16], and functional investigations exploring the collagen-binding site within the DDR1-DSD [17]. Together, these studies indicate that the four surface-exposed loops situated within the DSD combine to form an amphiphilic binding trench that binds to the GVMGFO motifs present in fibrillar collagens I-III [15,16,17]. Key residues that define this binding trench include the apolar residues W52, T56 & C73-C177 (Q136, L140 & C164-C266 in CPX-1) as well as the charged residues D69, R105 & E113 (D160, R192 & D199 in CPX-1) [16](Fig 2). Our sequence alignment revealed these key residues were highly conserved in CPX-1, and CPX-2 and ACLP. Furthermore, ACLP has been reported to associate with collagen I [18]. These observations prompted us to hypothesise that the CPX-1 DSD may serve as a collagen-binding domain.

CPX-1 binds collagen

To determine whether CPX-1 bound collagen we used an in vitro “pull-down” assay featuring agarose beads conjugated with either collagen III or IgG (control) and secreted CPX-1, concentrated from conditioned culture medium. CPX-1 was pulled down by the collagen but not the IgG control beads demonstrating that CPX-1 is able to bind collagen (Fig 3A – compare lanes 2 & 4 with lanes 3 & 5). Quantitation across multiple experiments revealed 15-20% of the recombinant CPX-1 bound under the assay conditions used.

N-glycosylation is not required for collagen binding

Having demonstrated that N-glycosylation of CPX-1 was essential for secretion we investigated whether N-glycosylation was required for collagen binding. To examine this we pre-treated secreted CPX-1 with PNGaseF prior to performing the pull-down assay. PNGaseF-treated CPX-1 bound collagen with similar efficacy to untreated CPX-1 (Fig 3B - compare lanes 2 & 5). These results suggest that N-glycosylation is not required for collagen binding although it is a prerequisite for CPX-1 secretion.

CPX-1 exists as monomer and dimer - only monomer binds collagen

DSDs are often found in pairs with some proteins containing a pair of DSDs, including the Coagulation Factors V and VIII and Neuropilin 1, whilst others contain a single DSD but form dimers, such as the DDRs. In the latter case dimerisation may be mediated by disulphide bonds, situated external to the DSD, and or interactions between the transmembrane domains and dimerisation appears to be critical for ligand binding and biological function [19,20]. To investigate whether CPX-1 may form disulphide-mediated dimers and, if so, whether dimerisation affected collagen binding we analysed secreted CPX-1 both pre and post collagen pull-down under non-reducing (-DTT) and standard reducing (+DTT) conditions. Analysis under non-reducing conditions demonstrated the presence of two major secreted CPX-1 species that migrated with mobility consistent with monomer and dimer (Fig 3C – lane 1). Quantitation of the proportion of monomer to dimer from 3 independent experiments indicated a ratio of 3 to 2. Perhaps surprisingly, only the CPX-1 monomer bound collagen (Fig 3C – compare lane 2 with lanes 1 & 3). These results demonstrate that CPX-1 is capable of forming disulphide-mediated dimers but only monomeric CPX-1 is able to bind collagen, suggesting dimerisation precludes collagen binding.

The DSD is required for CPX-1 binding to collagen

Finally, to investigate the putative role of the DSD in the CPX-1 – collagen binding interaction we generated and characterised a mutant form of CPX-1, termed CPX-1_{R192A}, where the conserved

R192 residue situated within the third loop of the DSD (see Fig 2) and integral to the binding trench [16] was mutated to Ala. A previous report showed that mutation of the equivalent residue in DDR1 (R105) reduced collagen binding by 80% [17]. We compared the expression, secretion and binding characteristics of CPX-1_{R192A} and wild-type (WT) CPX-1. The intracellular levels of CPX-1_{R192A} were comparable to those of the WT protein (Fig 4A). Secretion of CPX-1_{R192A} was compromised by 40% relative to WT (Fig 4B). Furthermore, compared with WT, collagen binding of CPX-1_{R192A} was reduced by 60% (Fig 4C). These results indicate that an intact DSD is required for efficient binding of CPX-1 to collagen and suggest that even conservative mutations within the DSD may compromise CPX-1 folding and secretion.

Discussion

Determining the cellular, molecular and biological characteristics and functions of proteins of unknown function represents a challenge that can be addressed using computational and biochemical methods [21]. In the current study we have employed such complementary approaches to show that CPX-1 is a secreted collagen-binding glycoprotein. We have shown that secretion of CPX-1 is dependent on N-glycosylation and that CPX-1 is secreted in both monomeric and dimeric forms. N-glycosylation of CPX-1 does not appear to be essential for collagen binding whilst only the monomer binds collagen. We also report that mutation of a key residue within the CPX-1 DSD (R192) reduced collagen binding providing direct evidence of a role for the DSD in this interaction. These observations provide a framework for future investigations exploring the biological function of CPX-1.

CPX-1 was identified as a novel member of the CPE subfamily that lacked catalytic activity [3]. Preliminary characterisation indicated that CPX-1 was a secreted glycoprotein that exhibited a relatively restricted expression profile prompting speculation that CPX-1 may be involved in developmental processes [3]. A subsequent report implicated CPX-1 in the process of osteoclastogenesis [9]. In the current report we first confirmed that CPX-1 is a secreted glycoprotein and then extended these observations demonstrating that efficient secretion of CPX-1 is dependent on N-glycosylation. This finding suggests that N-glycosylation of CPX-1 facilitates its appropriate folding and enables it to attain a secretion-competent conformation.

In silico analysis revealed 4 putative N-glycosylation sites. Notably, PNGaseF treatment of secreted CPX-1 did not restore CPX-1 to its 'ground state' of 80kDa, as was observed for intracellular CPX-1. One possibility is that secreted CPX-1 may comprise additional PNGaseF-insensitive PTMs. These may reflect fucose-linked α(1→3) N-glycans, which are resistant to the action of PNGaseF, or discrete PTMs. Another possibility is that secreted CPX-1 may be folded such that steric hindrance may preclude PNGaseF access to all N-glycan moieties. Additional studies are required to elaborate these details.

A focus of the current report was to establish a putative molecular role for CPX-1 by focusing on the DSD. Sequence alignment demonstrated conservation of around 50% with DSDs in related proteins, CPX-2 and ACLP, and around 30% with DSDs from non-related proteins, with the highest identity shared with the collagen-binding DSDs from the DDRs. More detailed analysis revealed conservation of key residues involved in mediating the DDR-DSD – collagen interaction via the formation of an amphiphilic binding trench [16,17]. These residues are also conserved in CPX-2 and ACLP, with the latter reported to associate with collagen I in studies implicating ACLP in pulmonary fibrosis [18]. This high degree of conservation prompted us to speculate that the CPX-1 DSD may serve as a collagen binding domain. Our *in vitro* binding studies, using collagen III, demonstrated that CPX-1 is able to bind collagen.

Further investigations indicated that removal of the PNGaseF-sensitive N-glycan(s) did not affect such binding. One of the putative N-glycosylation sites (N210) is situated within the DSD, between loops 3 and 4, at a site that is conserved in CPX-2 and ACLP. A putative N-glycosylation site is similarly located within the DSD of DDR2. However, detailed characterisation of DDR2 indicates this site is not glycosylated [5] and our modelling studies (not shown) suggest these residues are distinct from the binding trench. In contrast, a phospholipid-binding DSD within Factor VIII contains a validated N-glycosylation site [22] that, in combination with other N-glycans, facilitates binding [23]. Moreover, N-glycosylation of DDR1 at a residue distal to the DSD (N211), that is conserved in DDR2, has been shown to affect receptor activation [24]. Collectively these observations support the notion that glycosylation is unlikely to directly affect the DSD – collagen interaction *per se* but is likely to be important for modulation of conformation in the absence and or presence of collagen [5,24].

Dimerisation of the DDRs has consistently been reported to be essential for efficient collagen binding [19,20,25]. Studies of the extracellular domains of both DDR1 and DDR2 indicated that collagen binding required dimerisation [19,20] with transmembrane-interactions [25] and disulphide-bonds [19] proposed as key mediators of dimerisation. In the current report we showed that CPX-1 is secreted in both monomeric and dimeric form with the dimer being sensitive to reduction with DTT consistent with disulphide-mediated dimerisation. The DSD contains four conserved cysteines that, based on the DDR-DSDs [5], are likely to form two intramolecular disulphide bonds, one that links the N- and C-termini of the DSD (C115 & C274) and one that forms a disulphide bridge at the bottom of the collagen-binding trench (C164 & C266). There are seven additional cysteines present in CPX-1 and it seems likely that one or more of these are involved in the formation of disulphide-linked dimers. Notwithstanding, our results suggest that monomeric, but not dimeric, CPX-1 binds collagen. Whilst this contrasts with the scenario described above for the DDRs it is supported by observations, albeit indirect, where a recombinant monomeric form of the DSD from ACLP was shown to be sufficient to restore collagen-dependent function in ACLP$^{-/-}$ lung fibroblasts [18].

It is tempting to speculate that, like the DDRs, CPX-1, as well as CPX-2 and ACLP, binds to the GVMGFO motif common to the fibrillar collagens I-III [15,16,17]. Detailed investigations have established relative specificity with respect to DDR-binding to alternate, non-fibrillar collagens such as the collagens IV and X and have also revealed that such specificity is defined by non-conserved residues located at the periphery of the binding trench [26]. Such investigations provide a framework by which to elaborate the detailed collagen-binding properties of CPX-1, CPX-2 and ACLP. The molecular role of these proteins is, as with other DSD-containing proteins, likely to be determined by the nature of the DSD-binding partners. Although relatively little is known about CPX-1 or CPX-2, ACLP typically exhibits high levels of expression in collagen-rich tissues and emerging evidence suggests a collagen-associated role for ACLP in physiological and pathophysiological processes such as adipogenesis and pulmonary fibrosis [18,27], thereby highlighting novel potential therapeutic opportunities afforded by such understanding.

In summary, we have shown that CPX-1 is a secreted collagen-binding glycoprotein and, based on this and previous observations, we propose that the molecular function of the three

secreted, catalytically-inactive CPE subfamily of proteins, namely CPX-1, CPX-2 and ACLP, is likely to be elaborated, at least in part, by defining the molecular details of the respective DSD-collagen interaction.

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Figure Legends

Figure 1 – N-glycosylation of CPX-1 is required for secretion. (A) Schematic showing organisation of CPX-1 and location of putative N-glycosylation sites (stars) at residues 57, 210, 318 and 472. **(B)** CPX-1 was transiently expressed in CHO cells and intracellular (lysate) and secreted (media) CPX-1 was analysed by Western blot. **(C & D)** CPX-1 expressing CHO cells were treated with tunicamycin (Tuni - 10 μg/ml) for 24h or 3h and intracellular and secreted CPX-1 was analysed by Western blot. **(E & F)** Intracellular and secreted CPX-1 were treated with PNGaseF (2.5 units for 2h) and analysed by Western blot. Blots are representative of 3 independent experiments.

Figure 2 – In silico analysis of the DSD within CPX-1. Multiple sequence alignment of the DSD in human CPX-1, CPX-2, ACLP, DDR1, DDR2, Factor V, Neuropilin I and Factor VIII in decreasing order of homology. Loop regions (1-4) are boxed in grey. Conserved residues that form the collagen-binding trench are boxed in yellow. Putative N-glycosylation sites are underlined in green. The degree of conservation across all 8 DSDs is denoted as (*****) identical, (:) highly conserved and (.) conserved; Additional conservation across CPX-1/2, ACLP & DDR1/2 is denoted as (*, : and .) respectively. Numbers in parentheses depict % identity with CPX-1.

Figure 3 – CPX-1 binds collagen. (A) Secreted CPX-1 was incubated with collagen-agarose beads (col), or IgG-agarose beads (IgG) as control, and bound (B) and unbound (UB) material was

analysed by Western blot. ST – starting material. **(B)** As in (A) except samples were pre-treated with PNGaseF as indicated prior to incubation with the collagen-agarose beads. **(C)** As in (A) except following incubation with the collagen-agarose beads both bound and unbound samples were treated -/+ DTT prior to Western blot. Blots are representative of 3 independent experiments.

Figure 4 – Mutation of the CPX-1 DSD compromises collagen binding. (A & B) Wild-type CPX-1 (WT) and CPX-1_{R192A} (R192A) were transiently expressed in HEK cells and intracellular (cell lysates - **A**) and secreted (conditioned media - **B**) CPX-1 was analysed by Western blot. **(C)** Secreted WT and mutant CPX-1 were incubated with collagen agarose beads and bound (B) and unbound (UB) material was analysed by Western blot. Graphs show quantitation from 6 independent experiments. *$p<0.05$ WT cf R192A.

REFERENCES

[1] M.R. Sapio, L.D. Fricker, Carboxypeptidases in disease: insights from peptidomic studies, Proteomics Clin Appl 8 (2014) 327-337.

[2] S.E. Reznik, L.D. Fricker, Carboxypeptidases from A to z: implications in embryonic development and Wnt binding, Cell Mol Life Sci 58 (2001) 1790-1804.

[3] Y. Lei, X. Xin, D. Morgan, J.E. Pintar, L.D. Fricker, Identification of mouse CPX-1, a novel member of the metallocarboxypeptidase gene family with highest similarity to CPX-2, DNA Cell Biol 18 (1999) 175-185.

[4] X. Xin, R. Day, W. Dong, Y. Lei, L.D. Fricker, Identification of mouse CPX-2, a novel member of the metallocarboxypeptidase gene family: cDNA cloning, mRNA distribution, and protein expression and characterization, DNA Cell Biol 17 (1998) 897-909.

[5] F. Carafoli, E. Hohenester, Collagen recognition and transmembrane signalling by discoidin domain receptors, Biochim Biophys Acta 1834 (2013) 2187-2194.

[6] A. Kiedzierska, K. Smietana, H. Czepczynska, J. Otlewski, Structural similarities and functional diversity of eukaryotic discoidin-like domains, Biochim Biophys Acta 1774 (2007) 1069-1078.

[7] C.M. Borza, A. Pozzi, Discoidin domain receptors in disease, Matrix Biology 34 (2014) 185-192.

[8] B. Leitinger, Discoidin domain receptor functions in physiological and pathological conditions, Int Rev Cell Mol Biol 310 (2014) 39-87.

[9] E.J. Chang, H.B. Kwak, H. Kim, J.C. Park, Z.H. Lee, H.H. Kim, Elucidation of CPX-1 involvement in RANKL-induced osteoclastogenesis by a proteomics approach, FEBS Lett 564 (2004) 166-170.

[10] G.Y. Chuang, J.C. Boyington, M.G. Joyce, J. Zhu, G.J. Nabel, P.D. Kwong, I. Georgiev, Computational prediction of N-linked glycosylation incorporating structural properties and patterns, Bioinformatics 28 (2012) 2249-2255.

[11] R. Gupta, S. Brunak, Prediction of glycosylation across the human proteome and the correlation to protein function, Pac Symp Biocomput (2002) 310-322.

[12] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Research 32 (2004) 1792-1797.

[13] F.S. Newell, H. Su, H. Tornqvist, J.P. Whitehead, J.B. Prins, L.J. Hutley, Characterization of the transcriptional and functional effects of fibroblast growth factor-1 on human preadipocyte differentiation, FASEB J. 20 (2006) 2615-2617.

[14] J. Roth, C. Zuber, S. Park, I. Jang, Y. Lee, K.G. Kysela, V. Le Fourn, R. Santimaria, B. Guhl, J.W. Cho, Protein N-glycosylation, protein folding, and protein quality control, Mol Cells 30 (2010) 497-506.

[15] O. Ichikawa, M. Osawa, N. Nishida, N. Goshima, N. Nomura, I. Shimada, Structural basis of the collagen-binding mode of discoidin domain receptor 2, Embo J 26 (2007) 4168-4176.

[16] F. Carafoli, D. Bihan, S. Stathopoulos, A.D. Konitsiotis, M. Kvansakul, R.W. Farndale, B. Leitinger, E. Hohenester, Crystallographic insight into collagen recognition by discoidin domain receptor 2, Structure 17 (2009) 1573-1581.

[17] R. Abdulhussein, C. McFadden, P. Fuentes-Prior, W.F. Vogel, Exploring the collagen-binding site of the DDR1 tyrosine kinase receptor, J Biol Chem 279 (2004) 31462-31470.

[18] S.L. Schissel, S.E. Dunsmore, X. Liu, R.W. Shine, M.A. Perrella, M.D. Layne, Aortic carboxypeptidase-like protein is expressed in fibrotic human lung and its absence protects against bleomycin-induced lung fibrosis, Am J Pathol 174 (2009) 818-828.

[19] R. Abdulhussein, D.H. Koo, W.F. Vogel, Identification of disulfide-linked dimers of the receptor tyrosine kinase DDR1, J Biol Chem 283 (2008) 12026-12033.

[20] B. Leitinger, Molecular analysis of collagen binding by the human discoidin domain receptors, DDR1 and DDR2. Identification of collagen binding sites in DDR2, J Biol Chem 278 (2003) 16761-16769.

[21] C.L. Mills, P.J. Beuning, M.J. Ondrechen, Biochemical functional predictions for protein structures of unknown or uncertain function, Comput Struct Biotechnol J 13 (2015) 182-191.

[22] K.F. Medzihradszky, M.J. Besman, A.L. Burlingame, Structural characterization of site-specific N-glycosylation of recombinant human factor VIII by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry, Anal Chem 69 (1997) 3986-3994.

[23] M. Kosloski, R. Miclea, S. Balu-Iyer, Role of Glycosylation in Conformational Stability, Activity, Macromolecular Interaction and Immunogenicity of Recombinant Human Factor VIII, The AAPS Journal 11 (2009) 424-431.

[24] H.-L. Fu, R.R. Valiathan, L. Payne, M. Kumarasiri, K.V. Mahasenan, S. Mobashery, P. Huang, R. Fridman, Glycosylation at Asn211 Regulates the Activation State of the Discoidin Domain Receptor 1 (DDR1), Journal of Biological Chemistry 289 (2014) 9275-9287.

[25] N.A. Noordeen, F. Carafoli, E. Hohenester, M.A. Horton, B. Leitinger, A transmembrane leucine zipper is required for activation of the dimeric receptor tyrosine kinase DDR1, J Biol Chem 281 (2006) 22744-22751.

[26] H. Xu, N. Raynal, S. Stathopoulos, J. Myllyharju, R.W. Farndale, B. Leitinger, Collagen binding specificity of the discoidin domain receptors: binding sites on collagens II and III and molecular determinants for collagen IV recognition by DDR1, Matrix Biol 30 (2011) 16-26.

[27] A. Gusinjac, A. Gagnon, A. Sorisky, Effect of collagen I and aortic carboxypeptidase-like protein on 3T3-L1 adipocyte differentiation, Metabolism 60 (2011) 782-788.

Figure 1 - Single column

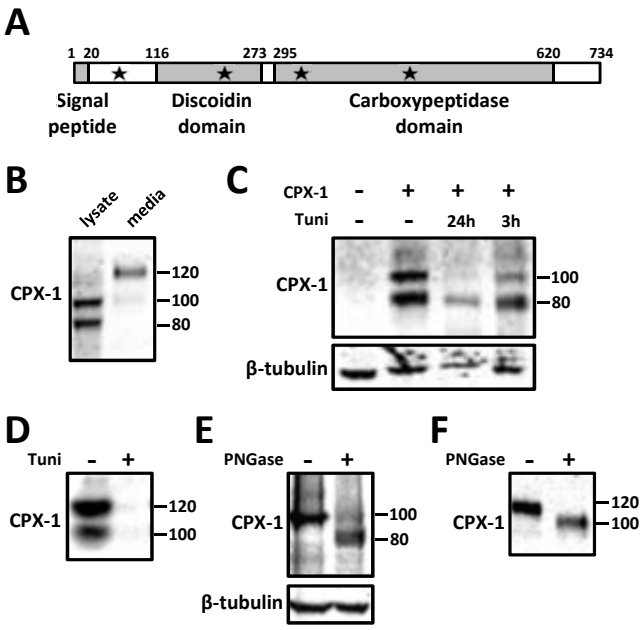


Figure 2 - Double column

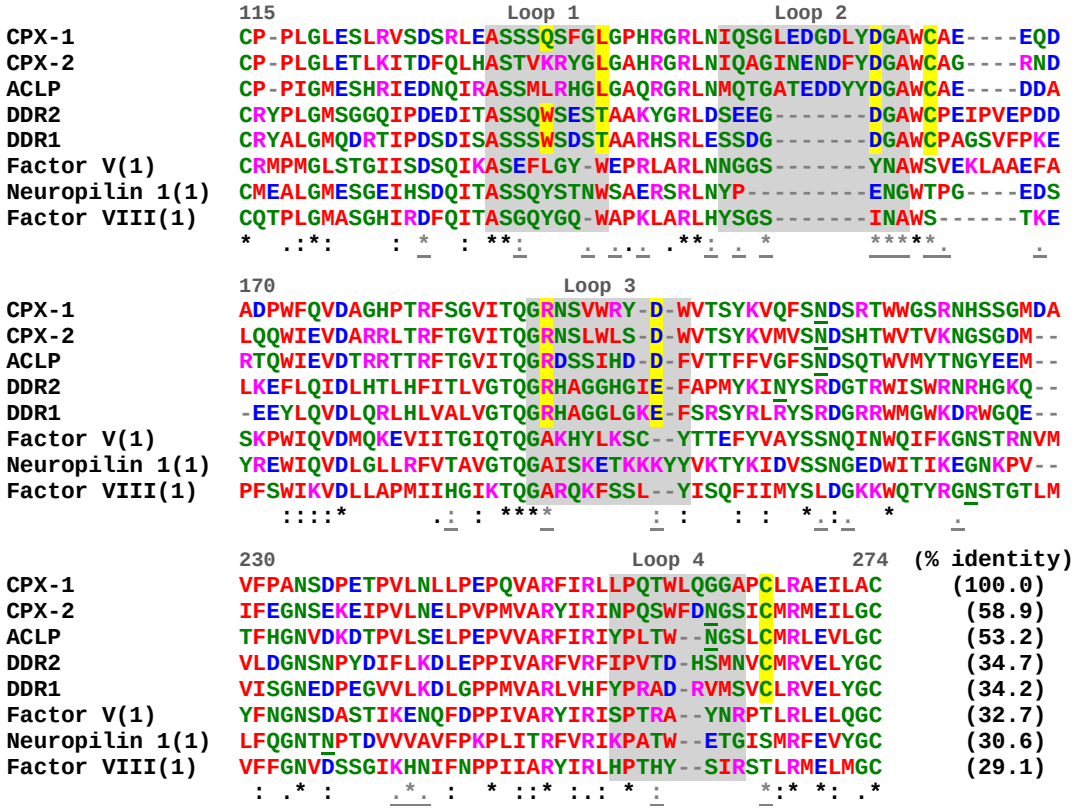


Figure 3 - Single column

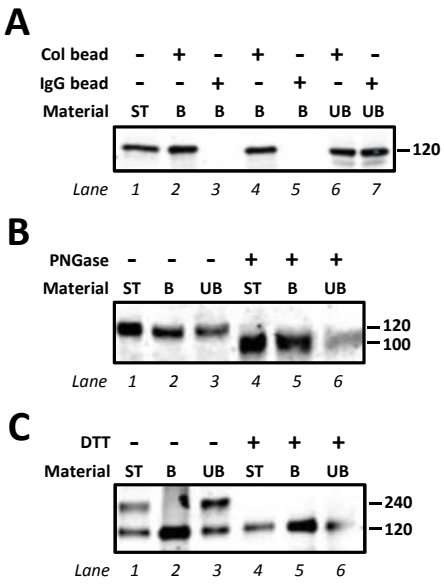
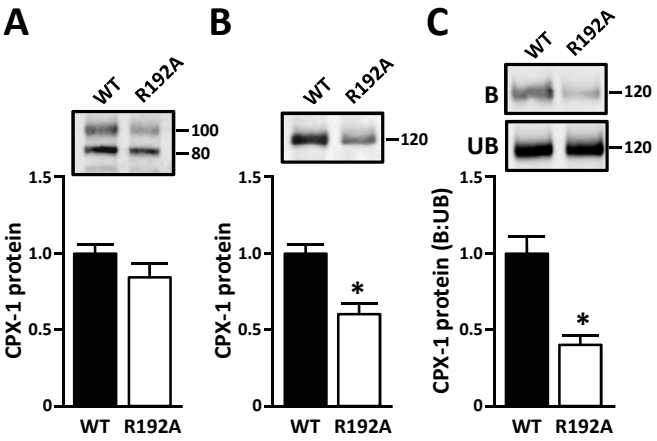


Figure 4 - Single column



Highlights

- The discoidin domain-containing protein CPX-1 is secreted
  - in an N-glycosylation dependent manner
  - in monomeric and dimeric forms
- CPX-1 binds collagen
  - in an N-glycosylation independent manner
  - in monomeric form only
- Mutation of the discoidin domain compromises collagen binding